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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12Q 1/68, G01N 33/53, 33/553, 33/567

A1

(11) International Publication Number: WO 96/09409

(43) International Publication Date: 28 March 1996 (28.03.96)

(21) International Application Number: PCT/US95/11971

(22) International Filing Date: 19 September 1995 (19.09.95)

(30) Priority Data:
08/309,229 20 September 1994 (20.09.94) US

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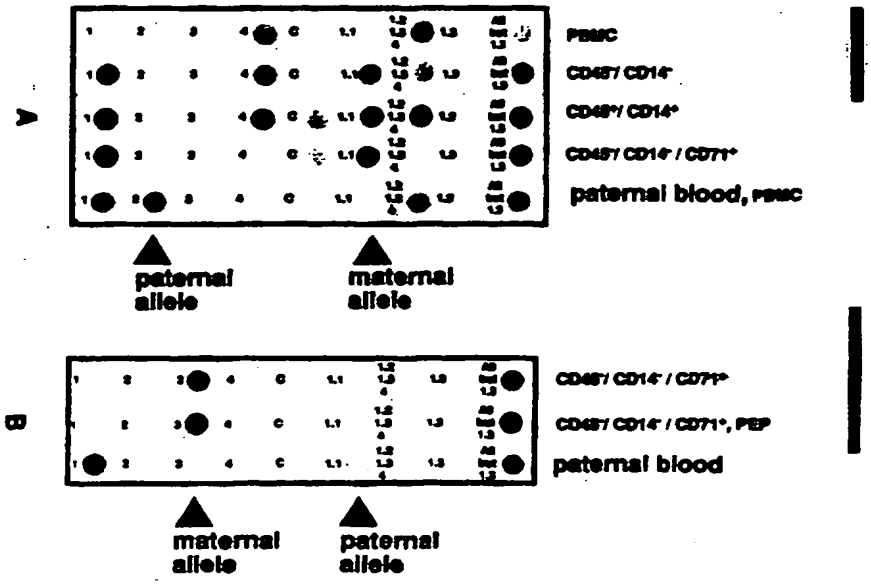
(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

Published
With international search report.

(54) Title: ENRICHMENT OF FETAL CELLS FROM MATERNAL BLOOD



(57) Abstract

Methods of preparing fetal cells from samples of maternal peripheral blood are provided. A suspension of mononuclear cells is prepared from a maternal blood sample. Maternal cells are then depleted from the sample by adding magnetically coupled antibodies specific for markers present on adult cells, followed by binding the cells to a column in the presence of a magnetic field. From the depleted fraction, fetal erythrocytes are enriched by magnetic cell sorting (MACS). These nucleated erythrocytes are then used as a source of fetal genetic material, which is analyzed for the presence of chromosomal or genetic abnormalities, or for the presence of a Y chromosome, by conventional methods such as probes for in situ hybridization, metaphase spreads, and polymerase chain reaction (PCR).

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Enrichment of Fetal Cells From Maternal Blood

INTRODUCTION

Technical Field

The field of the invention is prenatal diagnosis.

Background

The development of prenatal genetic testing has progressed rapidly in recent years. Knowledge of DNA gene sequences has led to a better understanding of what proteins or genes correspond to known diseases. Congenital diseases such as sickle cell anemia, muscular dystrophy, Tay-Sachs disease, Huntington's disease and Down's syndrome have been traced to specific gene mutations, or chromosomal abnormalities. It is now possible in many cases to determine before birth, by the use of molecular biological techniques, whether or not a fetus will have chromosomal or genetic defects.

Current techniques in molecular diagnosis require a sample of DNA from the cells of the developing fetus. Any source of fetal cells must be able to provide sufficient DNA for analysis. Those procedures that are currently used carry some degree of risk for the fetus. Amniocentesis and chorionic villus sampling are medically

accepted, but are invasive, and have a statistically significant chance of causing infection or miscarriage.

There has been much interest in the possibility of using fetal cells found in maternal blood as a source of fetal genetic material. Three types of nucleated fetal cells have been identified so far in maternal blood: placental trophoblasts, fetal erythroblasts and fetal lymphocytes. The observed frequencies of fetal cells in whole maternal blood range from 10^{-4} to 10^{-8}, which is too low to allow direct detection of fetal alleles by PCR.

Several authors have described the enrichment of fetal cells from maternal blood, using fluorescence-activated cell sorting (FACS) with monoclonal antibodies against CD71 (transferrin receptor), syncytiotrophoblastic cellular elements, polymorphic HLA class I antigens and CD71/anti-glycophorin A. However, the high technological effort required for FACS has prevented its routine use in medical diagnosis. FACS sorting is a time consuming and cost intensive procedure. FACS sorting has the additional disadvantage in that it is difficult to sort large numbers of cells, or to sort multiple samples at the same time.

An alternative approach to cell sorting has been described, whereby magnetic microparticles coupled to antibodies are used to select for specific cell types. Magnetic cell sorting (MACS) has been used to select for $CD71^+$ cells in maternal blood, however the level of enrichment was insufficient to detect fetal specific DNA by polymerase chain reaction. An improvement of the technique, adding a triple density gradient step, was reported to allow detection of fetal trisomies in the enriched cell fraction, however, technical difficulties in gradient stability have resulted in poor population purity with this method.

An improved MACS process whereby fetal cells could be sorted from maternal blood, and which allows multiple, and potentially large, samples to be run on the bench would provide numerous benefits to the field of prenatal diagnosis.

Relevant Literature

A review of prenatal diagnoses using maternal blood can be found in Chueh and Golbus (1990) Seminars in Perinatology **14**:471-482. The use of the fluorescence activated cell sorter to purify fetal cells away from maternal cells is described in Price *et al* (1991) Am.J.Obst.Gynecol., **65**:1731-1737; and Yeoh *et al.* (1991), Prenatal Diagnosis **11**:117-123. The presence and frequency of fetal nucleated cells in maternal peripheral blood is described in Hamada *et al.* (1993) Hum. Genet. **91**:427-432.

A number of methods for the detection of DNA sequences have been published. See, for example, Trask (1991) Trends in Genetics **7**:149-154; Wiley *et al.* (1984) In Vitro **20**:937-941; Lo *et al.* (1991) Nucleic Acid Research **19**:3561-3567; Erlich *et al.* (1991) Eur. J. Immunogenetics **18**:33-55; Kasai *et al.* (1980) Forensic Sci. **35**:1196-1200; and Budowle *et al.* (1991) Am. J. Hum. Genet. **48**:137-144.

Methods of enriching for nucleated red blood cells have been described in Bhat *et al.* (1993) J. Immunol. Methods **158**:277-280; Bianchi *et al.* (1993) Prenat. Diagnosis **13**:293-300; and Holgreve (1992) Am. J. Obstet. Gynecol. **166**:1350-1355.

High gradient magnetic cell sorting is described in Miltenyi *et al.* (1990) Cytometry **11**:231-238. The application of magnetic cell sorting to prenatal diagnosis is discussed in Velasco *et al.* (1992) AJRI **30**:194-201; and Smith (1993) J. Med. Genet. **30**:1051-1056.

SUMMARY OF THE INVENTION

Methods and compositions for the detection of fetal DNA sequences and chromosomal characteristics by non-invasive procedures are provided. Peripheral blood is drawn from the mother, desirably at a site distant from the developing fetus. Fetal nucleated cells are enriched from mononuclear cells of maternal blood, using a two step high-gradient magnetic cell separation (Double MACS). Maternal cells are depleted by specific binding to markers present on adult lymphoid and myeloid cells.

In a separate step, fetal cells are enriched by MACS. These nucleated erythrocytes are then used as a source of fetal genetic material, which is analyzed for the presence of chromosomal or genetic characteristics by conventional methods such as probes for *in situ* hybridization, metaphase spreads and polymerase chain reaction (PCR).

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B show the results of PCR analysis of the HLA-DQ A1 locus, using as a source of DNA: maternal peripheral blood mononuclear cells; $CD45^-/CD14^-$, $CD45^+/CD14^+$, and $CD45^-/CD14^-/CD71^+$ double MACS sorted cells from maternal blood; and peripheral blood mononuclear cells from paternal blood, analyzed with a commercially available HLA-DQ A1 typing kit. The DNA in Figure 2B was subjected to a PCR pre-amplification step, as indicated.

Figure 2 shows the results of PCR analysis of the D1S80 locus, using as a source of DNA: peripheral blood mononuclear cells; $CD45^-/CD14^-$, $CD45^+/CD14^+$ and $CD45^-/CD14^-/CD71^+$ double MACS sorted cells from maternal blood; and peripheral blood mononuclear cells from paternal and child blood.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Methods and compositions for the prenatal detection of fetal DNA sequences and chromosomal characteristics are provided. Peripheral blood is drawn from the mother. A preparation is then made of nucleated cells from the maternal blood. Fetal erythrocytes are enriched from the maternal cells by a combination process involving depletion of maternal cells and enrichment of fetal cells, using high-gradient magnetic cell separation (Double MACS), or a combination of high and low gradient magnetic separation. A suspension of blood cells are labeled with superparamagnetic particles specific for cell surface antigens, then sorted by binding to magnetic columns. The double-MACS procedure is most effective when a two-stringency system is used,

where the depletion step captures a high percentage of labeled cells and the enrichment step captures a lower percentage of labeled cells. The sorted fetal cells are used as a source of genetic material, which is analyzed to determine fetal sex, or the determination of other genetic characteristics.

The use of high-gradient magnetic cell sorting (MACS) to enrich for fetal cells provides several benefits when compared to flow cytometry methods presently used today, particularly for clinical practice. The subject methods require inexpensive reagents and apparatus, which are easily used and maintained. By setting up multiple columns, many samples can be processed at the same time. An automated system can be used to simplify processing of large sample numbers. The very low frequency of fetal cells in maternal circulation favors the use of relatively large maternal blood samples, which cannot easily by sorted with flow cytometry.

The mother will be at least about 6 gestational weeks (g.w.), usually at least about 8 g.w. or later in pregnancy. Generally the mother will be from about 8 to 20 g.w., although analysis may be performed later if required. The maternal blood sample is drawn from any site, conveniently by venipuncture. The sample is usually at least about 20 ml, more usually at least about 40 ml and may be as large as about 500 ml, more usually not more than about 250 ml. The blood is treated by conventional methods to prevent clotting, such as the addition of EDTA, heparin or acid-citrate-dextrose solution.

A preparation of nucleated cells is made from the sample. Any procedure which can separate nucleated cells from adult erythrocytes is acceptable. The use of Ficoll-Paque density dradients or elutriation is well documented in the literature. Alternatively, the blood cells may be resuspended in a solution which selectively lyses adult erythrocytes, *e.g.* ammonium chloride potassium, ammonium oxalate, *etc.*

The sample of nucleated peripheral blood cells (NPBC) is selectively depleted of maternal cells. Depletion reagents attached to superparamagnetic particles are bound to cell surface antigens which are present on adult hematopoietic cells, but are low or

absent on fetal erythrocytes. Especially useful depletion reagents are antibodies against cell surface antigens. Whole antibodies may be used, or fragments, *e.g.*, Fab, $F(ab')_2$, light or heavy chain fragments, *etc*. Such antibodies may be polyclonal or monoclonal and are generally commercially available or alternatively, readily produced by techniques known to those skilled in the art. Antibodies selected for use in depletion will have a low level of non-specific staining, and will usually have an affinity of at least about 100 μM for the antigen.

A cocktail of depletion reagents will be used, in order to deplete a wide range of blood cell types. Generally, at least about 95% of adult nucleated peripheral blood cells will be bound by the cocktail of depletion reagents, more usually at least about 99%, and preferably at least about 99.5%. Suitable antigens for depletion include CD45 which is widely expressed on lymphoid cells; CD14 which is found on monocytes; CD34 which is expressed on progenitor cells, and CD15 which is primarily found on granulocytes. Other useful cell surface antigens include CD11, CD44, CD46, CD48, CD43, CD49d, CD3, CD19, CD56, CD7 and CD5. In a preferred embodiment, a cocktail of antibodies specific for CD14, CD45 and optionally CD34 are used. Other useful combinations of markers for cocktail formulation are CD43 and CD19; CD3, CD19, CD56 and CD15; CD7 and CD19; and CD43 and CD5.

The depletion reagent antibodies are coupled to superparamagnetic particles, prepared as described in U.S. Patent nos. 4,452,773 and 4,230,685. The microparticles will usually be less than about 100 nm in diameter, and usually will be greater than about 10 nm in diameter. The exact method for coupling is not critical to the practice of the invention, and a number of alternatives are known in the art. Direct coupling attaches the antibodies to the particles, as described in co-pending patent application no. 08/252,112, herein incorporated by reference. Indirect coupling can be accomplished by several methods. The depletion reagent antibodies may be coupled to one member of a high affinity binding system, *e.g.* biotin, and the particles attached to the other member, *e.g.* avidin. One may also use second stage antibodies which

recognize species-specific epitopes of the depletion antibodies, *e.g.* anti-mouse Ig, anti-rat Ig, *etc.* Indirect coupling methods allow the use of a single magnetically coupled antibody species with a variety of depletion antibodies.

A preferred method uses hapten-specific second stage antibodies coupled to the superparamagnetic particles, as described in co-pending patent application no. 08/252,112. The hapten specific antibodies will usually have an affinity of at least about 100 μM for the hapten. The depletion antibodies are conjugated to the appropriate hapten. Suitable haptens include digoxin, digoxigenin, FITC, dinitrophenyl, nitrophenyl, *etc.* Methods for conjugation of the hapten to antibody are known in the art.

While not necessary for practice of the subject methods, it may be useful to label the depletion antibodies with a fluorochrome, e.g. phycoerythrin, FITC, rhodamine, Texas red, allophycocyanin, *etc.* The fluorochrome label may be used to monitor microscopically or by flow cytometry the cell composition after the depletion and enrichment steps. Fluorescent labeling may conveniently utilize the same indirect coupling system as the magnetic particles. For example, a cocktail of digoxigenin-coupled depletion antibodies may be used in combination with anti-digoxigenin antibody coupled to magnetic particles, followed by labeling with a fluorochrome conjugated antibody directed to the anti-hapten antibody.

The depletion reagent antibodies are added to a suspension of NPBC, and incubated for a period of time sufficient to bind the available cell surface antigens. The incubation will usually be at least about 5 minutes and usually less than about 30 minutes. It is desirable to have a sufficient concentration of antibodies in the reaction mixture, so that the efficiency of the magnetic separation is not limited by lack of antibody. The appropriate concentration is determined by titration. The medium in which the cells are separated will be any medium which maintains the viability of the cells. A preferred medium is phosphate buffered saline containing from 0.1 to 0.5% BSA. Various media are commercially available and may be used according to the

nature of the cells, including Dulbecco's Modified Eagle Medium (dMEM), Hank's Basic Salt Solution (HBSS), Dulbecco's phosphate buffered saline (dPBS), RPMI, Iscove's medium, PBS with 5 mM EDTA, *etc.*, frequently supplemented with fetal calf serum, BSA, HSA, *etc.*

Where a second stage magnetically coupled antibody is used, the cell suspension may be washed and resuspended in medium as described above prior to incubation with the second stage antibodies. Alternatively, the second stage antibody may be added directly into the reaction mix. When directly coupled depletion antibodies are used, the cell suspension may be used directly in the next step, or washed and resuspended in medium.

The suspension of magnetically labeled cells is applied to a column or chamber as described in WO 90/07380, herein incorporated by reference. The matrix may consist of closely packed ferromagnetic spheres, steel wool, wires, magnetically responsive fine particles, *etc.* The matrix is composed of a ferromagnetic material, *e.g.* iron, steel, *etc.* and maybe coated with an impermeable coating to prevent the contact of cells with metal. The matrix should have adequate surface area to create sufficient magnetic field gradients in the separation chamber to permit efficient retention of magnetically labeled cells. The volume necessary for a given separation may be empirically determined, and will vary with the cell size, antigen density on the cell surface, cell number, antibody affinity, *etc.*

In order to maximize the purity of the final cell preparation, a two stringency system is employed, where the depletion step captures a high percentage of labeled cells and the enrichment step captures a lower percentage of labeled cells. This reduces the probability that labeled cells will be carried over from the first separation step into the second. The stringency of the depletion column will be such that at least about 95% of the labeled cells will be retained on the column in the presence of a magnetic field, usually at least about 99% of the labeled cells will be retained, and preferably at least about 99.9% retained. The geometry, matrix composition, magnetic field strength, size

and flow rate of the ferromagnetic column will determine the percent of labeled cells that are retained on the column. Factors that will increase the stringency are increased column size and length, decreased flow rate, and a finer matrix composition. A column matrix of fibers with fine particles is preferred for the depletion step. An empirical determination of the stringency may be made by analysis of bound and unbound cells.

The labeled cells are bound to the matrix in the presence of a magnetic field, usually at least about 100 mT, more usually at about 500 mT, usually not more than about 2T, more usually not more than about 1T. The source of the magnetic field may be a permanent or electromagnet. The unbound cells contained in the eluate are collected as the eluate passes through the column. For greater purity, the unbound cells may be passed a second time over the magnetic column.

The unbound cells are used in an enrichment step, to select for fetal nucleated cells. Enrichment reagents attached to superparamagnetic particles are bound to cell surface antigens which are present on fetal cells. Of particular interest is the use of reagents specific for the transferrin receptor, CD71, or other cell surface markers specifically present on fetal cells, particularly fetal liver derived erythrocytes. Antibody directly coupled to the superparamagnetic particle is preferred. CD71 is present on both adult and fetal activated cells, and is expressed at high levels on fetal erythrocytes. Contamination from CD71 expression on adult hematopoietic progenitor cells may be avoided by the use of CD34 in the depletion reagent cocktail.

Another marker of interest is glycophorin A, which is expressed on both adult and fetal erythrocytes. The possibility of contaminating adult red blood cells does not preclude the use of glycophorin A, as the adult cells are enucleated. However, care must be taken in the depletion step to include a reagent which recognizes adult reticulocytes.

The enrichment reagents, superparamagnetic particles, columns and buffers are prepared as described for the depletion reagents, however, the stringency for the enrichment column will be lower than for the depletion column. The stringency of the

enrichment column will be such that at least about 50% of the labeled cells will be retained on the column in the presence of a magnetic field, usually at least about 80% of the labeled cells will be retained, usually not more than 95% retained. A column matrix of spheres is preferred for the enrichment step. The cells are bound to the magnetic matrix. After the initial binding, the matrix is washed with any suitable physiological buffer to remove unbound cells. The unbound cells are discarded.

The bound cells are released by removing the magnetic field, and eluting in a suitable buffer. The cells may be collected in any appropriate medium which maintains the viability of the cells. Various media are commercially available and may be used according to the nature of the cells, including dMEM, HBSS, dPBS, RPMI, PBS-EDTA, PBS. Iscove's medium, *etc.*, frequently supplemented with fetal calf serum, BSA, HSA, *etc.*

In many cases the separation procedure will perform the depletion step first, followed by the enrichment step. If the enrichment step is to be performed first, then an additional step is necessary after the enrichment, in order to remove the magnetic label from the enriched cells. This may be accomplished by any suitable method. The enriched cell population may be incubated with a solution of dextranase, where the dextranase is present at a concentration sufficient to remove substantially all microparticles from the labeled cells. Usually the reaction will be complete in at least about 1 hour. The depletion step may then be performed as previously described with the dextranase treated cells.

Alternatively, the enrichment step may be performed first, and the depletion step modified to use large magnetic spheres in place of the microparticles. The use of such magnetic spheres has been previously described, and the reagents are commercially available. The enriched cell population is incubated with highly magnetic polymer spheres of about 5 to 10 μm diameter conjugated to the depletion antibody cocktail. The mixture of cells is then placed in close proximity to a magnetic field. Substantially all cells bound to the polymer spheres are bound to the magnet after about 1 minute,

and after not more than about 5 minutes. The unbound cells may be decanted and used for analysis.

After the depletion and enrichment steps are complete, the cells are used as appropriate. The method of harvesting will depend on the type of analysis to be performed. For those procedures which require purified DNA samples the cells will be lysed according to conventional methods. There are a number of suitable procedures described in the literature. If the analysis will be performed on whole chromosomes the cells will be suitably prepared according to conventional methods. In one preferred embodiment, the cells are immobilized on a filter, and are then labeled *in situ* with suitable fluorochrome coupled reagents.

The choice of genetic analysis is not critical to the invention. Any conventional method of analysis which requires fetal cells, DNA or chromosomes can be used. Examples include the use of polymerase chain reaction for detection of specific DNA sequences, fluorescence *in situ* hybridization for chromosome number, metaphase spreads for detection of chromosome complement and Quinicrin mustard staining to detect the presence of the Y chromosome.

The absolute number of fetal cells present in maternal circulation is small, and so methods which utilize the polymerase chain reaction (PCR) are of particular interest. DNA is isolated from the cell sample, and PCR used to amplify a region of DNA by the use of specific primers. The amplified DNA is then analyzed for the presence of specific alleles. The analysis may fractionate the DNA according to size to determine fragment length polymorphisms, or may use hybridization to determine the absence or presence of a specific sequence. In most cases, a pre-amplification step (PEP) with specific or non-specific primers may be used. Bulk PCR, where DNA from a number of cells is amplified in a single reaction is particularly useful to determine whether paternal alleles are present in the fetus, *e.g.* Y chromosome alleles, or genes for which the maternal and paternal alleles can be distinguished.

It is advantageous in the genetic analysis to discriminate between fetal cells and contaminating maternal cells. This may be accomplished by DNA mosaic detection. Isolated single cells are amplified by PCR, usually in combination with a pre-amplification step. The DNA is amplified and analyzed for the presence of the sequence of interest, and for a paternally derived marker. Any polymorphism which can be assigned to the paternal parent may be used. Of particular interest are polymorphic alleles of the major histocompatibility complex, *e.g.* HLA-A, HLA-B, HLA-DP, HLA-DQ and HLA-DR, microsatellite repeat polymorphisms, e.g. D9S52, APOC2, D19S49, D1S80, D8S320, SE33 *etc.*, polymorphisms in protein sequences, *etc.* Only those samples which differ from the maternal pattern are scored, thereby excluding any contaminating maternal cells.

A variation of DNA mosaic detection may also be used in combination with FISH. In addition to a fluorochrome labeled probe specific for the sequence of interest, a probe with a second fluorochrome for a paternally derived marker, or a fetal specific marker, *e.g.* Hemoglobin F, is added. Only those cells which which differ from the maternal pattern are scored.

Conditions which can be analysed by the subject methods include fragile X; trisomies of chromosome 21, 18, 12, 13, X and Y; and other chromosomal abnormalities; presence of fetal Y chromosome; and detecting the presence of genes correlated with such diseases as Tay-Sachs, muscular dystrophy, cystic fibrosis, hemophilias, and hemoglobinapathies, *e.g.* sickle cell anemia, thalassemias, *etc.*

In order to address the needs of a clinical laboratory, a kit may be provided having the reagents and apparatus necessary to perform the subject invention. Such a kit may contain for the depletion step: hapten conjugated marker specific antibodies, *e.g.* anti-CD34, anti-CD45, anti-CD14, *etc.*; anti-hapten antibody conjugated to superparamagnetic particles; and column(s) suitable for high stringency selection. Components which may be included for the enrichment step include superparamagnetically coupled marker specific antibody, *e.g.* anti-CD71, anti-

glycophorin A, *etc.*; and column(s) suitable for low stringency selection. Where required, large magnetic beads may be included. For convenience, buffers may be included for lysis of adult erythrocytes, cell staining and collection, *etc.* While single columns may be used, it is anticipated that multiple columns will be run simultaneously, and an apparatus for automated or manual procedures may be provided for such a purpose. Reagents for genetic analysis may also be included, particularly primers for PCR amplification, staining reagents for FISH and filters for cell immobilization.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Double MACS Enrichment of Fetal Erythrocytes from Maternal Blood

Materials and Methods

Isolation of mononuclear cells from peripheral blood

20 ml of blood was obtained from the arm vein of pregnant women and their husbands and 5% EDTA was added. The mononuclear cells and nucleated red blood cells from maternal blood were isolated by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation (600g for 20 min. at 20° C). The cells were washed twice with PBS (phosphate-buffered saline: 137 mM sodium chloride, 2 mM potassium chloride, 8 mM disodium phosphate, 1.5 mM potassium dihydrogen phosphate, pH 7.4).

Immunofluorescent and magnetic labeling

2×10^7 peripheral blood mononuclear cells (PBMC) as prepared above were resuspended in 150 µl of PBS with 0.01% sodium azide and 1% bovine serum albumin

(PBS/BSA/NaN_3) and stained with 200 µl of phycoerythrin conjugated murine anti-human CD45 monoclonal antibodies (KC 56 IgG1 Coulter, Krefeld, FRG) diluted 1 to 4 from the stock in PBS/BSA, incubated on ice for 10 min., washed once in PBS/BSA/NaN_3, and labelled with 150 µl of PBS/BSA/NaN_3 containing 30 µl MACS rat anti mouse IgG1-conjugated superparamagnetic microbeads and 30 µl anti-CD14-conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, FRG), at 4°C for 15 min. In some experiments digoxigenin coupled murine anti-human CD45 monoclonal antibodies and digoxigen coupled anti-CD14 antibodies were used in combination with anti-digoxigenin conjugated superparamagnetic particles. The labelled cells were washed once with PBS and applied to a MACS-A2 column (Miltenyi Biotec).

The negative fraction of the first MACS sort was stained again with 170 µl of fluorescein isothiocyanate (FITC) conjugated murine anti human CD71 mAb (12 µg/ml; LO1.1, IgG2a, Becton Dickinson, San Jose, CA, USA) in PBS/BSA/NaN_3 on ice for 10 min. The cells were washed and labelled with 30 µl MACS rat anti mouse IgG2a+b-conjugated superparamagnetic microbeads (Miltenyi Biotec) in 60 µl PBS/BSA/NaN_3 as described above. The cells were then washed and sorted by MACS. CD71 conjugated to superparamagnetic particles were purchased from Miltenyi Biotec.

Cell sorting with MACS

For the first MACS, aimed at depletion of CD45 and CD14 - expressing cells, the labelled cells were applied to an A2 column and sorted essentially as described by Miltenyi *et al.*, *supra*. For optimal depletion, the flow rate was kept to approx. 0.4 ml/min. by using a 26G needle at the outlet of the MACS column. The cells were then washed off with 1 ml of buffer. For the second MACS, which aimed at the isolation of $CD71^+$ cells from the overall low number of $CD45^-/CD14^-$ cells a MiniMACS column was used. The cells were labeled with CD71 conjugated microbeads. Negative cells were washed off the column at flow rates of approx. 0.35 ml/min. in a volume of 1 ml.

The column was then washed again four times with 0.5 ml of buffer. Finally, the $CD45^-/CD71^+$ cells were eluted from the column outside of the magnet in 1 ml of $PBS/BSA/NaN_3$. The number of live cells was evaluated just before and after loading the MACS column in the depletion and enrichment step by a Neubauer counter chamber. Dead cells were excluded by trypan blue staining.

Flow cytometry

All sorting steps were analyzed by five parameter three-color cytometric analysis with a FACScan flow cytometer (Becton Dickinson, San Jose, CA). 10,000 events per sample were recorded and analyzed using FACScan research software (Becton Dickinson). Live cells were gated according to scatter and relative propidium iodide to phycoerythrin staining, with propidium iodide (PI) specifically staining for dead cells (Fig 1A and B, Weichel et al., 1992). The factors of enrichment and depletion were calculated as follows:

$$\text{enrichment factor} = {}^{1}\!/_{\text{depletion factor}} = \frac{\%\ \text{neg. in orig. sample}}{\%\ \text{pos. in orig. sample}} \times \frac{\%\ \text{pos. in pos. fraction}}{\%\ \text{neg. in pos. fraction}}$$

$$\text{and the recovery is given as: } = \frac{\text{abs. number of target cells in positive fraction}}{\text{abs. number of target cells in original fraction}}$$

Extraction of DNA and PCR

DNA was extracted by standard procedures with alkaline lysis. DNA concentration was quantified UV-photometrically at 260 nm (Beckman UV-spectrometer SE 550).

HLA-DQ A1 genotyping was carried out using the Ampli-Type HLA-DQa kit (Cetus, Emeryville CA) according to the manufacturer's instructions, and a Perkin-Elmer thermocycler (Perkin-Elmer, Ueberlingen, FRG). Before specific HLA-DQ A1 amplification, the DNA was in some cases preamplified, using nonspecific primers for the primer-extension preamplification technique (PEP) described by Zhang *supra*.

PCR amplification of sequences from the D1S80 locus was carried out with the following primers:

A [SEQ ID NO:1]: 5'-GTCTTGTTGGAGATGCACGTGCCCCTTGC-3'

B [SEQ ID NO:2]: 5'-GAAACTGGCCTCCAAACACTGCCCGCCG-3'

(Kasai *et al. supra*).

The cycling conditions for the D1S80 PCR were as follows: denaturation at 94°C for 1 min. after hot start, annealing at 67°C for 1 min., extension at 72°C for 1 min, 30 cycles. PCR products were separated on discontinuous native polyacrylamide gels (stacking gel: 3.5% T, 2.7% C, resolving gel: 7%T, 4% C). DNA fragments were detected by silver staining. Alleles differed in size and were classified with the help of the "allelic ladder" of the D1S80 typing kit (Perkin-Elmer). To determine the ability of the HLA-DQA1 and D1S80 PCRs to detect specific alleles, we titrated DNA from one heterozygous donor into DNA from another heterozygous donor. Both donors shared one allele. We compared this titration to a titration of DNA from sorted $CD45^-/CD71^+$ cells. The relative concentration at which no more PCR signal was obtained, i.e. the limit of sensitivity, was compared to that concentration of DNA from $CD45^-/CD71^+$ cells, at which no signal was obtained for the paternal allele. At this point the known frequency of the specific allele in the allelic mixture was considered equal to the frequency of the paternal allele at that dilution of DNA.

RESULTS

Purification of $CD45^-/CD71^\pm$ cells by MACS

Mononuclear cells were obtained from 20 ml of peripheral blood from 11 pregnant women (weeks 12 to 25 of gestation). $CD45^+$ leukocytes and in some cases $CD14^+$ cells were magnetofluorescently labelled (71 - 92% of cells gated according to scatter) and depleted on a MACS column. The negative fraction contained 0.6 - 2% stained cells. This fraction was magnetically stained again for CD71 and applied to a second MACS. The eluted fraction contained 62 to 87% of $CD71^+$ cells. Both MACS sortings together required approximately 30 minutes of time.

Table 1
Efficiency of CD45/CD14 depletion and CD71 enrichment by Double MACS

Patient	$CD45^+ / 14^+$ before MACS 1*		$CD45^+ / 14^+$ after MACS 1 depletion		$CD45^+/14^+$ Depletion factor	$CD45^- / 14^-CD71^+$ before MACS 1*		$CD45^-/14^-CD71^+$ after MACS 1		$CD45^- / 14^- CD71^+$ after MACS 2 enrichment		$CD45^-/14^- CD71^+$ Enrichment factor	$CD45^-/14^- CD71^+$ Recovery
	(%)	No.	(%)	No.		(%)	No.	(%)	No.	(%)	No.		(%)
1	89.1	$1.6x10^7$	0.83	$1.6x10^4$	977	0.61	$1.1x10^5$	4.45	$8.9x10^4$	83.3	$4.5x10^4$	813	41.3
2	72.3	$1.2x10^7$	0.85	$4.4x10^4$	476	1.12	$1.9x10^5$	2.84	$1.5x10^5$	87.5	$8.1x10^4$	618	42.6

Numbers of live cells from 20 ml maternal blood were evaluated by a Neubauer counter chamber.
* = Cell numbers are determined after staining and just before loading the MACS column.

Table 1 shows the results of two separations. A simple MACS $CD71^+$ enrichment from ficoll separated PBMC is compared with a double-MACS $CD45^-/CD14^-/CD71^+$ enrichment of the same sample. The $CD71^+$ cell population (1.2%) was 9.4 fold enriched after the CD45/CD14 cell depletion (10.2%) and another 32.1 fold by the subsequent $CD71^+$ enrichment of the Double-MACS procedure, leading to a total enrichment of 301fold (78.5%). The direct $CD71^+$ enrichment of the MACS procedure yielded an enrichment rate of only 33 fold (28.9%).

In all 11 separations, the $CD45^+/CD14^+$ cells were depleted from 72 - 92% to 0.6% - 2.0% (depletion rates of 242 - 977, average 780). In the second MACS these remaining $CD45^-/CD14^-$ cells were again enriched to 1.9% - 4.8% among the eluted cells. $CD45^-/CD71^+$ cells were enriched from 0.5 - 2.1% to 1.4 - 10.2% (enrichment rates of 2.8 - 9.4, average 3.9) in the first MACS by depletion of CD45/CD14 cells and in the second MACS, the $CD45^-/CD71^+$ cells were enriched to 62 - 87% (enrichment rates of 31 - 465, average of 130). Thus the overall enrichment rates for $CD45^-/CD71^+$ cells varied between 300 and 820 with an average of 500. Recovery rates varied between 38% - 55%. The sorted cells were analyzed by flow cytometry, and DNA was prepared from them for genetic analysis.

PCR analysis of HLA-DQ A1 and D1S80 polymorphism

DNA from unsorted and sorted maternal mononuclear cells, paternal cells and in one case from infant cells was subjected to PCR to detect the presence of specific HLA-DQ alleles, and D1S80 fragment length polymorphism. In the experiment shown in Fig 1 (12th week of gestation), an allele inherited from the father (allele 2) was detected in the $CD45^-/CD14^-/CD71^+$ cells, but not in unsorted PBMC, the $CD45^-/CD14^-$ or the $CD45^+/CD14^+$ fraction. Some DNAs were preamplified with the primer-extension preamplification technique (PEP) described by Zhang *et al.* (1993) P.N.A.S. 89:5847-5851. In another sample, taken from a woman in the 13th week of pregnancy, a

paternal allele was detected in the fetal cells only after PEP preamplification and HLA-DQ A1 genotyping.

With the D1S80 PCR paternal alleles were detected only in the $CD45^-/CD14^-/CD71^+$ fraction, but not in unsorted PBMC or in sorted $CD45^+/CD14^+$ cells, shown in Figure 2. After birth the genotype of the child was verified.

Paternal alleles were detected in fetal cells from maternal blood in 7 out of 11 analyzed cases. For HLA DQ A1 genotyping the success rate of detection was 6 out of 11 (Table 2). In one sample, mother and father shared a HLA DQ A1 allele (1.2) and no allelic difference between mother and fetus was demonstrated. It is not clear whether the fetus had inherited the shared allele from the father.

With regard to D1S80 genotyping, in one sample mother and father shared a D1S80 allele (T22), making the detection of a fetal allele impossible. For D1S80 polymorphism fetal alleles were detected in 3 out of 11 samples. In these samples fetal DQ A1 alleles were also detected. In 3 out of 11 samples D1S80 PCR products from DNA of maternal cells were not observed.

Frequencies of fetal $CD45^-/CD71^+$ cells in maternal blood

The relative frequencies of fetal cells in maternal blood by PCR of HLA DQ A1 and D1S80 was estimated by comparing a titration of $CD45^-/CD71^+$ cells to a titration of cells representing the paternal genotype into cells representing the maternal genotype. The D1S80 PCR was able to detect specific allelic length polymorphism with a limit of sensitivity of 1 paternal allele in 20 maternal alleles. The HLA-DQ-A1 PCR showed a higher sensitivity for detection of a particular allele in the presence of an excess of other different alleles. The sensitivity ranged from 1 in 100 (without PEP) to 1 in 200 (with PEP).

For $CD45^-/CD71^+$ cells enriched from maternal blood by Double-MACS, titration of DNA to the limit of detectability of paternal genes by PCR was consistent with relative frequencies of paternal to maternal alleles of 1 in 20 to 1 in 200. The

average amount of DNA obtained from the sorted $CD45^-/CD71^+$ cells of 20 ml of maternal blood was 175 ng, as evaluated photometrically. We obtained 0.9 to 8.75 ng fetal DNA from cells isolated from 20 ml maternal blood, corresponding to 130-1300 $CD45^-/CD71^+$ fetal cells in 20 ml of maternal blood. This corresponds to a frequency of fetal cells among maternal cells of 10^{-5} to 10^{-6} in PBMC, before MAC Sorting.

It is evident from the above results that the subject invention provides for a simple, fast method for separating fetal nucleated erythrocytes from maternal blood. The subject methods are non-invasive, and so do not carry the risk of infection and miscarriage that come with amniocentesis and chorionic villus sampling. The ease of operation, and ability to scale up the number and size of samples, provide significant benefits over existing methods.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Miltenyi Biotech, Inc.

(ii) TITLE OF INVENTION: Enrichment of Fetal Cells from Maternal Blood by High Gradient Magnetic Sorting

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
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(F) ZIP: 94111-4187

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT/US95/
(B) FILING DATE: September 20, 1995
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(B) REGISTRATION NUMBER: 36,677
(C) REFERENCE/DOCKET NUMBER: A-59472/BIR/PJS

(ix) TELECOMMUNICATION INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GTCTTGTTGG AGATGCACGT GCCCCTTGC 29

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAAACTGGCC TCCAAACACT GCCCGCCG 28

WHAT IS CLAIMED IS:

1. A method for non-invasive prenatal testing to determine a genetic characteristic of the fetus, said method comprising:

depleting adult hematopoietic cells from a blood sample from a pregnant mother by the steps comprising:

preparing a suspension of nucleated cells from said blood sample;

adding magnetically coupled antibodies specific for at least one cell surface antigen expressed by adult hematopoietic cells and absent on fetal erythrocytes to said suspension of mononuclear cells;

passing said suspension of mononuclear cells through a ferromagnetic matrix in the presence of a magnetic field; and

collecting cells which are unbound to said ferromagnetic matrix to provide a depleted cell sample;

enriching for fetal erythrocytes in said depleted cell sample by the steps comprising:

adding magnetically coupled antibodies specific for a cell surface antigen expressed by said fetal erythrocytes to a suspension of said depleted cells;

passing said suspension of mononuclear cells through a ferromagnetic matrix in the presence of a magnetic field;

washing said matrix of unbound cells; and

eluting said fetal erythrocytes from said matrix in the substantial absence of said magnetic field to provide an enriched cell sample;

analyzing the genetic material of said enriched cell sample for said fetal genetic characteristic.

2. A method according to Claim 1, wherein said at least one cell surface antigen expressed by adult hematopoietic cells is selected from the group consisting of CD45, CD14, CD44, CD46, CD48, CD34 and CD11.

3. A method according to Claim 1, wherein said cell surface antigen expressed by said fetal erythrocytes is selected from the group consisting of CD71 and glycophorin A.

4. A method according to Claim 1, wherein said analyzing of genetic material comprises the steps of:

preparing DNA from said enriched cell sample;

amplifying a specific region of said DNA by polymerase chain reaction;

determining the presence of a paternal sequence in said amplified DNA.

5. A method according to Claim 4, wherein said paternal sequence is present on the Y chromosome.

6. A method for non-invasive prenatal testing to determine a genetic characteristic of the fetus, said method comprising:

depleting adult hematopoietic cells from a blood sample from a pregnant mother by the steps comprising:

preparing a suspension of mononuclear cells from said blood sample;

adding a cocktail of magnetically coupled antibodies specific for CD71 and for CD14 to said suspension of mononuclear cells;

passing said suspension of mononuclear cells through a ferromagnetic matrix in the presence of a magnetic field; and

collecting cells which are unbound to said ferromagnetic matrix to provide a depleted cell sample;

enriching for fetal erythrocytes in said depleted cell sample by the steps comprising:

adding magnetically coupled antibodies specific for CD71 to said depleted cell sample;

passing said suspension of mononuclear cells through a ferromagnetic matrix in the presence of a magnetic field;

washing said matrix of unbound cells; and

eluting said fetal erythrocytes from said matrix in the substantial absence of said magnetic field to provide an enriched cell sample;

preparing DNA from said enriched cell sample;

amplifying a specific region of said DNA by polymerase chain reaction;

determining the presence of a paternal sequence in said amplified DNA.

7. A kit for non-invasive prenatal genetic testing, comprising:

two columns of a ferromagnetic matrix;

a cocktail of magnetically coupled antibody specific for CD45 and for CD14;

magnetically coupled antibody specific for CD71.

Figure 1

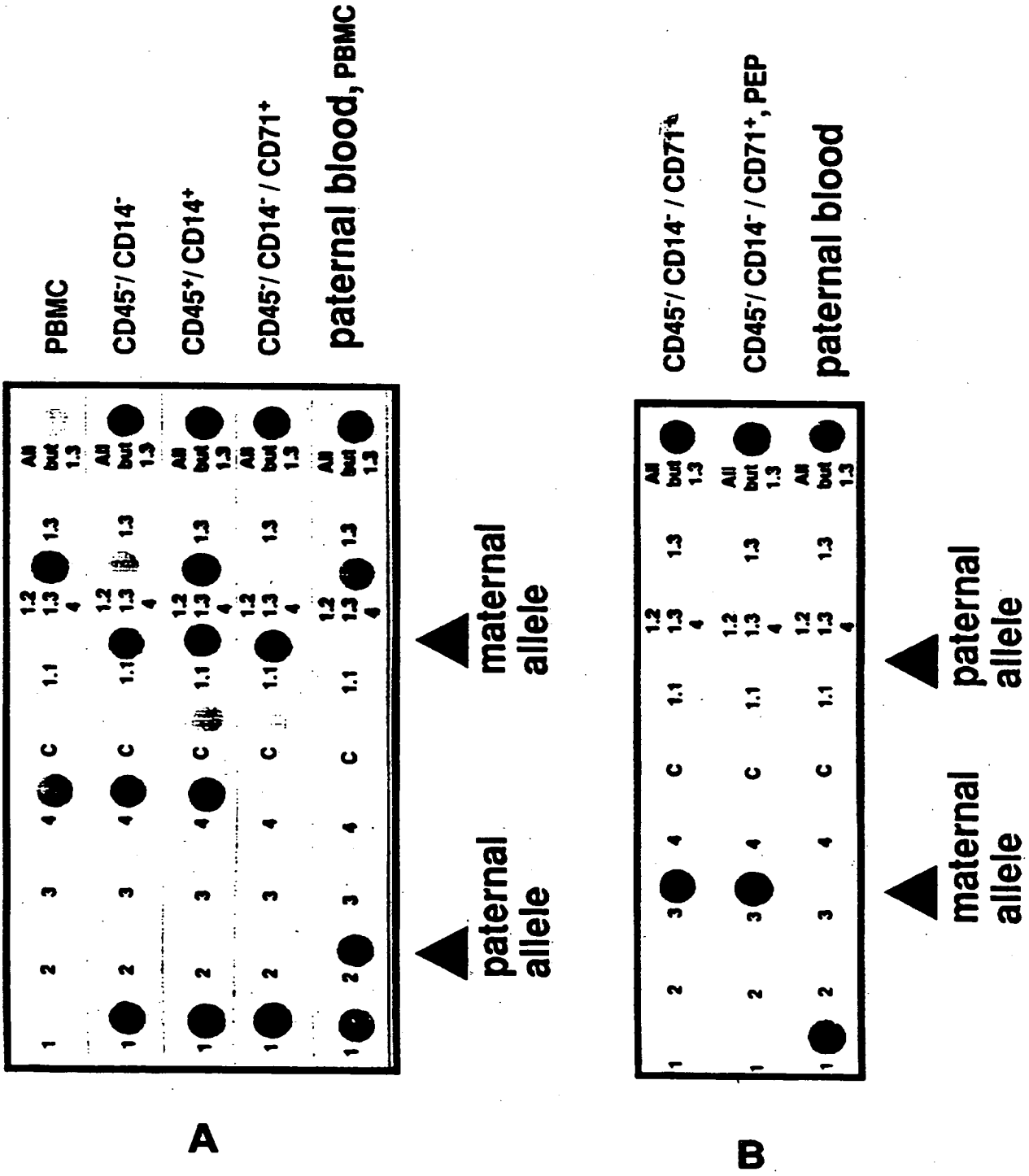
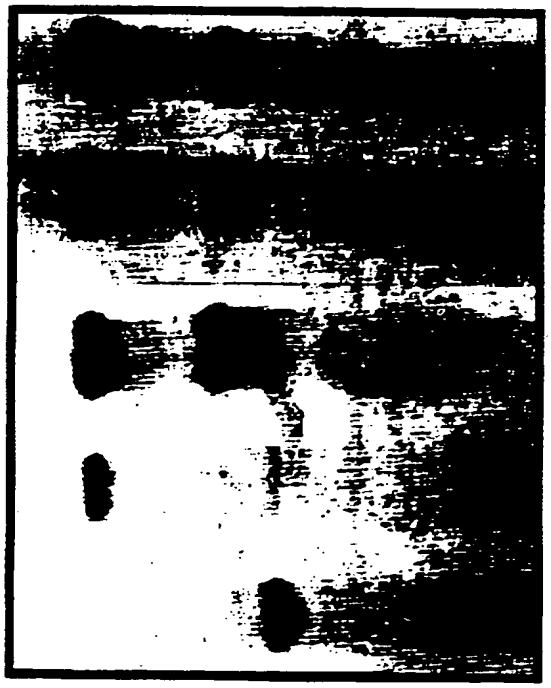

PBMC
CD45⁻/ CD14⁻
CD45⁺/ CD14⁺
CD45⁻/ CD14⁻ / CD71⁺
paternal blood, PBMC
maternal allele
paternal allele
A
CD45⁻/ CD14⁻ / CD71⁺
CD45⁻/ CD14⁻ / CD71⁺, PEP
paternal blood
paternal allele
maternal allele
B

Figure 2



PBMC

CD45+/ CD14+

CD45-/ CD14- / CD71+

child, PBMC

father, PBMC

mother, 21st week of gestation

maternal allele

paternal allele

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/11971

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C12Q 1/68; G01N 33/53, 33/553, 33/567
US CL :435/6, 7.24, 7.25; 436/526, 510
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.24, 7.25, 975; 436/526, 510

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, APS
search terms: maternal blood, fetal cell?, prenatal diagnosis, immunoselect?, cell sort?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	PRENATAL DIAGNOSIS, Volume 14, Number 12, issued December 1994, J. Büsch et al, "Enrichment of Fetal Cells From Maternal Blood by High Gradient Magnetic Cell Sorting (Double MACS) for PCR-Based Genetic Analysis", pages 1129-1140, see entire document.	1-7
X	ANALYTICAL CELLULAR PATHOLOGY, Volume 6, Number 3, issued 1994, J. Büsch et al, "Simple and Fast 'Double MACS' Sorting of Fetal Cells From Maternal Blood for PCR-Based Analysis During the Early Gestation", page 289, Abstract #335, see entire Abstract.	1-7

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
30 NOVEMBER 1995

Date of mailing of the international search report
05 JAN 1996

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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/11971

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF MEDICAL GENETICS, Volume 30, Number 12, issued December 1993, Y. Zheng et al, "Prenatal Diagnosis From Maternal Blood: Simultaneous Immunophenotyping and FISH of Fetal Nucleated Erythrocytes Isolated by Negative Magnetic Cell Sorting", pages 1051-1056, see entire document, especially page 1052.	1-7
Y	AMERICAN JOURNAL OF REPRODUCTIVE IMMUNOLOGY, Volume 30, Numbers 2-3, issued 1993, D. Gänshirt-Ahlert et al, "Detection of Fetal Trisomies 21 and 18 From Maternal Blood Using Triple Gradient and Magnetic Cell Sorting", pages 194-201, see entire document.	1-7
Y	FETAL DIAGNOSIS AND THERAPY, Volume 7, Numbers 3-4, issued 1992, V. Cacheux et al, "Detection of 47, XYY Trophoblast Fetal Cells in Maternal Blood by Fluorescence In Situ Hybridization After Using Immunomagnetic Lymphocyte Depletion and Flow-Cytometry Sorting", pages 190-194, see entire document.	1-7
Y	PRENATAL DIAGNOSIS, Volume 13, Number 4, issued April 1993, D.W. Bianchi et al, "Erythroid-Specific Antibodies Enhance Detection of Fetal Nucleated Erythrocytes in Maternal Blood", pages 293-300, see entire document.	1-7
Y	AMERICAN JOURNAL OF OBSTETRICS AND GYNECOLOGY, Volume 165, Number 6, issued December 1991, J.O. Price et al, "Prenatal Diagnosis with Fetal Cells Isolated From Maternal Blood By Multiparameter Flow Cytometry", pages 1731-1737, see entire document.	1-7